MTBC Detection Kit v1

USER MANUAL

For *in vitro* Diagnostic Use

Document Code: MB05v2f
Approval Date: July 2011

IVD CE

Contents

	Page
1. Product Description	1
2. Content	1
3. Storage	1
4. Required Materials and Devices	1
5. Important Notes and Safety Instructions	2
6. Product Use Limitations	2
7. Pathogen	2
8. Method	3
9. Procedure	4
9.1. Sample Preparation, Storage and Transport	4
9.2. Interfering Substances	4
9.3. DNA Isolation	4
9.4. Kit Components	4
9.4.1. PCR Mix	4
9.4.2. Detection Mix 1	4
9.4.3. Detection Mix 2	4
9.4.4. Internal Control	4
9.4.5. Positive Control	5
9.5. Preparing the PCR	5
9.6. Programming the Montania® 483 Real-Time PCR Instrument	5
10. Analysis	7
11. Troubleshooting	9
12. Specifications	10
12.1. Sensitivity	10
12.2. Cross-Reactivity	10
12.3. Reproducibility and Precision	10
13. References	11
14. Symbols	11
15. Contact Information	11

1. PRODUCT DESCRIPTION

Bosphore® MTBC Detection Kit v1 detects MTBC DNA in human biological samples, encompassing all the complex MTBC genotypes (M. tuberculosis, M. bovis, M. bovis BCG, M. africanum, M. canetti, M. caprae, M. microti and M. Pinnipedi). The analytic sensitivity is 128 copies/ml. A region within the 16S ribosomal RNA gene is amplified and fluorescence detection is accomplished using the FAM filter.

An internal control has been integrated into the kit in order to check PCR inhibition. The amplification data of the internal control is detected with the Cy5 filter. The internal control is added during PCR step.

2. CONTENT

Bosphore® MTBC Detection Kit v1 is composed of Real-Time PCR reagents, an internal control and a positive control.

Component	REAGENT	100 Tests	50 Tests	25 Tests
1	dH_2O	(1000 µl)	(1000 µl)	(500 µl)
2	PCR Mix	(2240 µl)	(1120 µl)	(560 µl)
3	Detection Mix1	(176 µl)	(88 µl)	(44 µl)
4	Detection Mix2	(132 µl)	(66 µl)	(33µl)
5	Internal Control	(23µl)	(15 µl)	(15 µl)
6	Positive Control 1	(160 µl)	(80 µl)	(40 µl)

3. STORAGE

Bosphore® MTBC Detection Kit v1 PCR reagents should be stored at -20°C. Repeated thawing and freezing (>3x) should be avoided since it may reduce sensitivity. If the components are to be used in small amounts, they should be frozen in aliquots.

While preparing the PCR; the components should not be exposed to room temperature for more than 10 min. and the detection mix components should not be exposed to light more than 1-2 min. We recommend preparing the PCR on a cooling block and keeping the detection mixes within a closed container.

The components maintain their stability until the expiry dates on the labels, if they are stored at advised conditions.

4. REQUIRED MATERIALS AND DEVICES

- Montania® 483 Real-Time PCR Instrument (Anatolia Geneworks), or another Real-Time PCR system with FAM and Cy5 filters (iCycler, iQ5, CFX–BioRad, LightCycler 1.5, 2.0, 480-Roche, 7500 Real-Time PCR System-ABI, Stratagene Mx3005P, Mx3000P-Agilent, LineGeneK, LineGene 9600-Bioer, Rotorgene 2000, 3000, 6000, Q-Qiagen)
- 0.2 ml Thin-Wall PCR tubes or strips
- Magnesia® 16 Nucleic Acid Extraction System / Magnesia® Bacterial DNA Extraction Kit (Anatolia Geneworks) or other high quality DNA extraction kits and systems
- Deep freezer (-20°C)
- Desktop centrifuge with rotor for 2 ml. microcentrifuge tubes
- Calibrated adjustable micropipettes
- DNAse, RNAse, pyrogen free micropipette tips with filters
- DNAse, RNAse, pyrogen free 1.5 or 2 ml. microcentrifuge tubes
- Disposable laboratory gloves

5. IMPORTANT NOTES AND SAFETY INSTRUCTIONS

Important!:

- The product should be delivered on dry ice. Check for presence of dry ice upon arrival.
- Check for the expiry dates on the box and tube labels, upon arrival. Do not use expired products or components.
- Calibrated or verified micropipettes, DNAse, RNAse, pyrogen free micropipette tips with filters, and DNAse, RNAse, pyrogen free microcentrifuge tubes should be used.
- Before starting a test procedure, all components should be thoroughly thawed. After thawing, all components should be centrifuged briefly (spin-down for 3-5 seconds), and mixed well to ensure homogeneity prior to use.
- The kit components should be kept on ice or a cooling block until the reaction is prepared, and they should be quickly returned to -20°C.
- PCR and nucleic acid isolation must be performed in different compartments. Samples should be stored separately to avoid contact with the kit components.
- Pathogen information should be reviewed to be aware of the health related risks.
- Clinical samples should be handled with extreme caution: Physical contact with pathogens should be avoided by; wearing lab coats and gloves, no allowance for eating or drinking within the workspace, prevention of unauthorized individuals' access to the working area.
- All the pathogenic wastes produced during the nucleic acid isolation step; including the biological samples and material contacted with them, should be discarded into medical waste and disposed safely.

6. PRODUCT USE LIMITATIONS

- All the components may exclusively be used for in vitro diagnostics.
- This product should be used in accordance with this user manual.
- This product is to be used by personnel specially trained to perform in vitro diagnostic procedures.

7. PATHOGEN

Causative Agents

MTBC (Mycobacterium Tuberculosis Complex) contains seven closely related bacteria species with distinct host tropisms including M. tuberculosis, M. bovis, M. bovis BCG, M. africanum, M. canetti, M. caprae, M. microti and M. Pinnipedi. The most common member of the complex is the M. Tuberculosis. The human pathogens *M. africanum* and *M. canettii* and species adapted to animals *M. bovis* (bovine), *M. caprae* (goats), *M. pinnipedii* (seals), and *M. microti* (rodents) are the other members of the complex. Members of the MTBC are estimated as genetically monomorphic with a high level of genomic sequence similarity (>99.95%), limited horizontal gene transfer, and a clonal population structure. It is accepted as the genetic diversity among MTBC strains has not any clinical significance because of this apparent homogeneity. They cause tuberculosis in humans by localizing in the lungs.[1]

Epidemiology

Tuberculosis is the most common second infection worldwide causing the death of young and older people. One-third of the world population is infected with tuberculosis. Each year 9 million people worldwide have been infected with tuberculosis and nearly 2 million of this has resulted in death. In Turkey between 10 million and 20 million people are infected with tuberculosis and carry infectious agent of tuberculosis (mycobacterium tuberculosis). The prevalence of TB is approximately 27 in one hundred thousand. [2], [3]

Modes of Transmission

Even though it can affect all organs of the body, the main entry of the infection is respiratory system through lung. It is transmitted through air droplets which are generated when TB infected persons cough, sneeze, speak, etc. Also being in close contact with a person that has active TB disease with TB germs present in the sputum and consumption of unpasteurized infected cow's *milk* that has Mycobacterium bovis are the other causes of the human tuberculosis. [4]

8. METHOD

Bosphore® MTBC Detection Kit v1 is based on the Real-Time PCR method. Polymerase chain reaction is a technique that is used for amplification of a DNA region. The reaction occurs by the repeating cycles of heating and cooling. The main components of PCR are primers, dNTPs, Taq polymerase enzyme, buffer solution and template. As a brief explanation, primers are small synthetic DNA those anneal to the specific regions of the template in order to start the synthesis. dNTPs are the building blocks of the amplified products. Taq polymerase amplifies the DNA template. Buffer solution provides the pH adjustment required for the reaction and template, as referred, is the target region for synthesis.

In Real Time PCR technique, in contrast to conventional PCR, PCR product can be monitored during the reaction. Therefore Real-Time PCR obviates the need for further analysis methods like gel electrophoresis, whereby minimizing the risk of contamination. Dual labeled probes employed in the reaction in addition to the conventional PCR reagents, enable detection of the amplified target with increased sensitivity.

The assay utilizes the 5′ exonuclease activity of Taq Polymerase to cleave a dual-labeled fluorescent hybridization probe during the extension phase of PCR.

The probe is labeled at the 5′ end with a fluorescent 'reporter' molecule, and at the 3'end with another fluorescent molecule that acts as a 'quencher' for the 'reporter'. When the two fluorophores are in close proximity, and the reporter is excited by light, no reporter fluorescence can be detected. During the elongation step of PCR, Taq Polymerase encounters and cleaves the probe bound to the template. As the reporter is freed from the suppressing effect of the quencher, fluorescence signal can be detected.

The fluorescence generated by the reporter increases as the PCR product is accumulated; the point at which the signal rises above background level and becomes distinguishable, is called the threshold cycle (C_T). There is a linear relationship between the log of the starting amount of a template and its threshold cycle.

Bosphore MTBC Detection Kit v1 employs multiplex PCR, and an internal control is incorporated into the system in order to check for possible PCR inhibition. MTBC DNA and an internal control are co-amplified in a single reaction, using sequence-specific primers. The fluorescent signal generated by the MTBC amplification is detected by a probe labeled at the 3′ end with FAM, through the FAM channel. The fluorescent signal generated by the internal control amplification, is detected by a second probe (labeled at the 5′ end with a different reporter molecule, Cy5) through the Cy5 channel.

9. PROCEDURE

9.1. Sample Preparation, Storage and Transport

Human respiratory samples such as bronchoalveolar lavage (BAL), sputum, bronchial mucosa should be collected. To isolate bacterial DNA from the clinical specimen, great care should be taken to avoid exogenous contamination with mycobacterium.

All steps of DNA extraction must be performed under a biological safety cabinet. In order to ensure safety, samples might be processed in a decontamination procedure, if desired. On the other hand, decontamination procedures might reduce the sensitivity of the assay.

Specimen should be stored at 4°C for short-term (24 hours) if it is to be processed in a day or frozen at -20°C for longer periods of time. Sensitivity of the PCR might be reduced because of repeated cycles of freeze-thaw. DNase/RNAse free polypropylene tubes must be used for storage.

The samples should be transported in containers with capacity to resist pressure. Transportation should be done according to local and national regulations for pathogen material transport.

9.2. Interfering Substances

To avoid possible influences on PCR:

- Samples which have been collected in waxed containers or in propylene glycol test tubes
- Food particles or mouthwash
- Decontamination reagents such as isoniazid might reduce PCR sensitivity.

must not be used.

9.3. DNA Isolation

We recommend that the Magnesia® 16 Nucleic Acid Extraction System / Magnesia® Bacterial DNA Extraction Kit (Anatolia Geneworks) isolation system or an alternative high quality DNA extraction system is used with Bosphore® MTBC Detection Kit v1. The DNA isolation should be performed according to the manufacturers' instructions. The starting volume is 200 µl, the elution volume is 100 µl.

9.4. Kit Components

9.4.1. PCR Mix

HotStarTaq DNA Polymerase: HotStarTaq DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus,* cloned into E.Coli. The enzyme is provided in an inactive form. It is activated by a 15-minute 95 °C incubation step. This prevents the formation of misprimed products and primer-dimers during reaction setup and the first denaturation step, leading to high PCR specificity and accurate qualitative analysis. QuantiTect Probe PCR Buffer: contains Tris-Cl, KCl, $(NH_4)_2SO_4$, 8 mM $MgCl_2$, pH 8.7 (20°C). dNTP Mix: Contains ultrapure quality dATP, dGTP, dCTP ve dTTP/dUTP.

9.4.2. Detection Mix 1

Detection Mix 1 contains MTBC-specific forward and reverse primers and a dual-labeled probe.

9.4.3. Detection Mix 2

Detection Mix 2 contains internal control-specific forward and reverse primers and a dual-labeled probe.

9.4.4. Internal Control

An internal control is included in the kit to control PCR inhibition. The internal control is a synthetic DNA molecule derived from human genome. It is added directly into the PCR master mix to control the PCR inhibition exclusively. For this purpose, 0.2 µl of internal control should be added for each reaction into the master mix. Lack of

internal control amplification in the FAM negative samples, may indicate a problem in PCR or PCR inhibition. In this case, PCR should be repeated. In samples that contain a high bacterial load, internal control can be suppressed and no increase of the signal is detected. Please use the table below for the interpretation of internal control data:

MTBC (FAM)	Internal Control (Cy5)	Interpretation
+	+	Sample positive
-	+	Sample negative
+	-	Sample positive
-	-	Repeat the test!

9.4.5. Positive Control

The positive control contains MTBC DNA. It can be included in the PCR to test the efficiency of the PCR exclusively. The threshold cycle for the positive control is given in the acceptance criteria table (Section 10. Analysis). Threshold cycles higher than the acceptance criteria may indicate an efficiency loss in the reaction.

9.5. Preparing the PCR

Positive control should be added into the PCR reaction together with the samples and the negative control (PCR-grade water) in order to deduce the positivity/negativity of the samples. Make sure that all the kit components are thawed before use. Refer to the table below for preparing the PCR. It is for only one reaction, multiply these values with the sample number to find the values required for the master mix. While preparing master mixes for more than 5 samples, an extra 10% should be added to the total sample number.

PCR Mix	20 µl
Detection Mix 1	1.6 µl
Detection Mix 2	1.2 µl
Internal Control	0.2 µl
Sample DNA	18 µl
Negative/Positive Control	
Total Volume	41.0 µl

Pipette 23 µl of the master mix into the PCR tubes or strips, and add 18 µl of DNA (sample/positive or negative control). Close the tube cap. Make sure that the solution in each tube is at the bottom of the tube. Centrifuge if necessary.

9.6. Programming the Montania® 483 Real-Time PCR Instrument

The thermal protocol for Bosphore® MTBC Detection Kit v1 is composed of an initial denaturation for activation the HotStarTaq DNA Polymerase, a two-step amplification cycle and a terminal hold. The real-time data is collected at the second step of the amplification cycle.

Initial denaturation	95°C	14:30 min.	
Denaturation	97°C	00:30 min.	} 50 cycles
Annealing and Synthesis (Data Collection)	53°C	01:30 min.	
Hold	22°C	05:00	

Montania® 483 Real-Time PCR Instrument is installed and calibrated as it is delivered to the end user.

In order to establish an appropriate link between the system components, first the thermal cycler and the optical module, and then the PC and the software should be started.

Before starting a Real-Time PCR reaction using the Bosphore® Kits, the following steps should be completed:

- Choose the filter pairs to be used (FAM and Cy5).

- Identify unknown samples, positive and negative controls.
- Select the correct thermal protocol.

These steps are described below:

From the main menu of the Montania® 483 Real-Time PCR Instrument, "File" and then "New" is selected. "Create a new Experiment" is selected. In the "Select Channel" window channels 1 (FAM) and 3 (Cy5) are selected (Fig. 1). Samples positive and negative controls are identified in the "Module Edit" menu (Fig. 2). To select the thermal protocol "Gene Amplification" menu is used. The "Open" button in the "Experiment Program" is clicked and the appropriate thermal protocol is selected. (Fig. 3a). The thermal cycles of the selected protocol is displayed. The experiment starts by clicking the "Start" button (Fig. 3b).

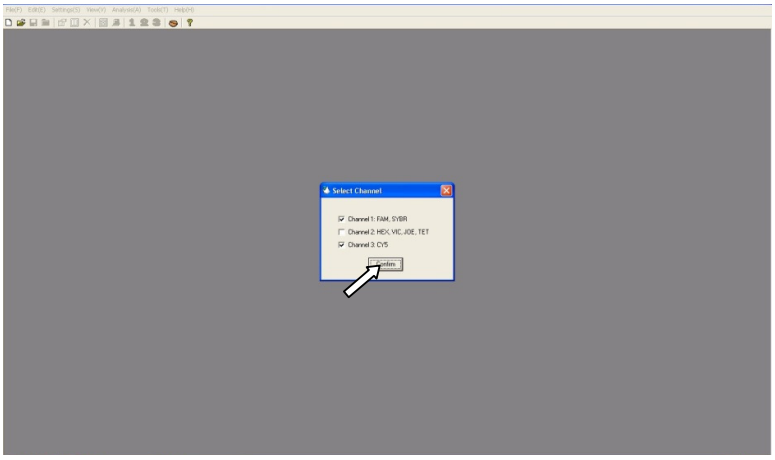

Fig. 1: Filter Selection in Montania® 483

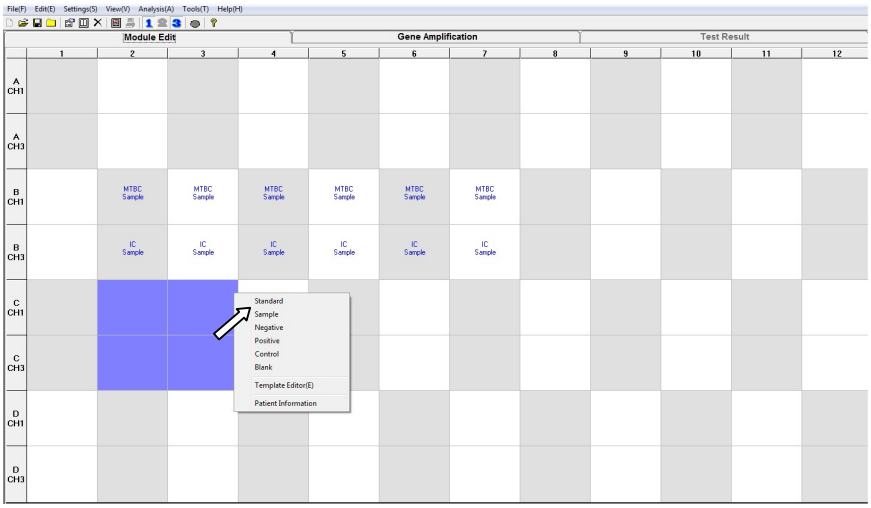

Fig. 2: Sample Location and Identification

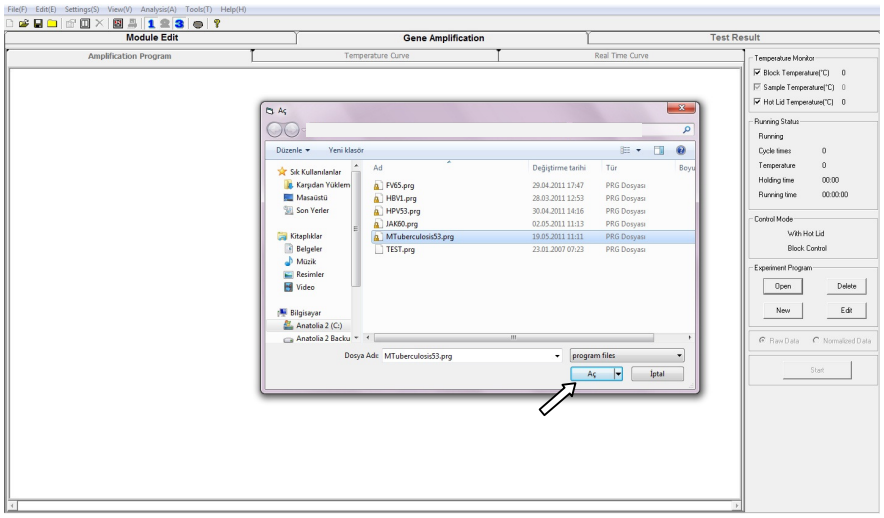

Fig. 3a: Selecting the Thermal Protocol

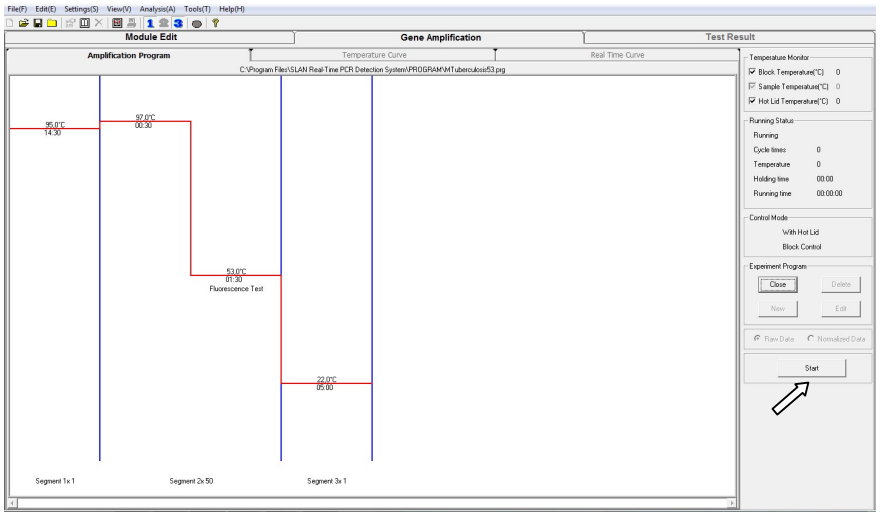

Fig. 3b: Starting the Experiment

10. ANALYSIS

By the end of the thermal protocol, the Montania® 483 Real-Time PCR Instrument software automatically calculates the baseline cycles and the threshold.

Example of an amplification curve is given in Fig. 4.

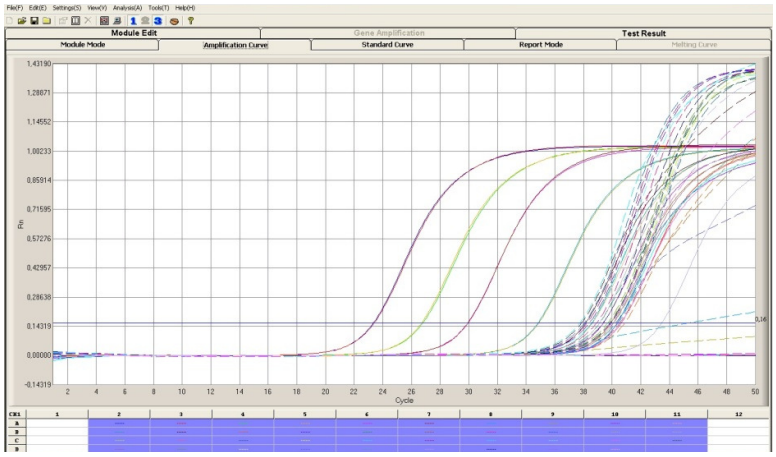

Fig. 4: Amplification Curve of a Bosphore® MTBC v1 test

Analysis of the results should be performed by trained personnel who have received the required training for analysing Real-Time PCR data. We recommend that the test results must be evaluated by an expert clinician, taking the patient's clinical findings and the results of other tests into consideration.

All analysis is done automatically in routine use. However, when the trained personnel, who have received the required training from manufacturer, consider it as necessary, the system allows pulling down the threshold as much as possible in order to detect low positive samples. In this case, attention should be paid to keep the threshold line above the background and to keep the correlation coefficient at the maximum possible value (and within its acceptance criteria.)

Positive control of Bosphore® MTBC v.1 is essential for accurate result analysis. The cycle threshold acceptance criteria for the positive control is 33±4 .

Test results should not be reported unless the assay results meet the criteria stated above. Please contact the manufacturer if impairment in the product's performance is observed (See the last page for contact information).

The qualitative results of the test are displayed on the "Report Mode" screen. The samples that cross the threshold in Channel 1 (FAM) are displayed as positive whereas samples that do not cut the threshold are displayed as "Negative" or "No Ct". These samples are regarded as negative or having a bacterial load below the detection limit of the assay. For these undetectable samples, the Cy5 data of the internal control should also be checked to avoid false negative results (Fig. 6).

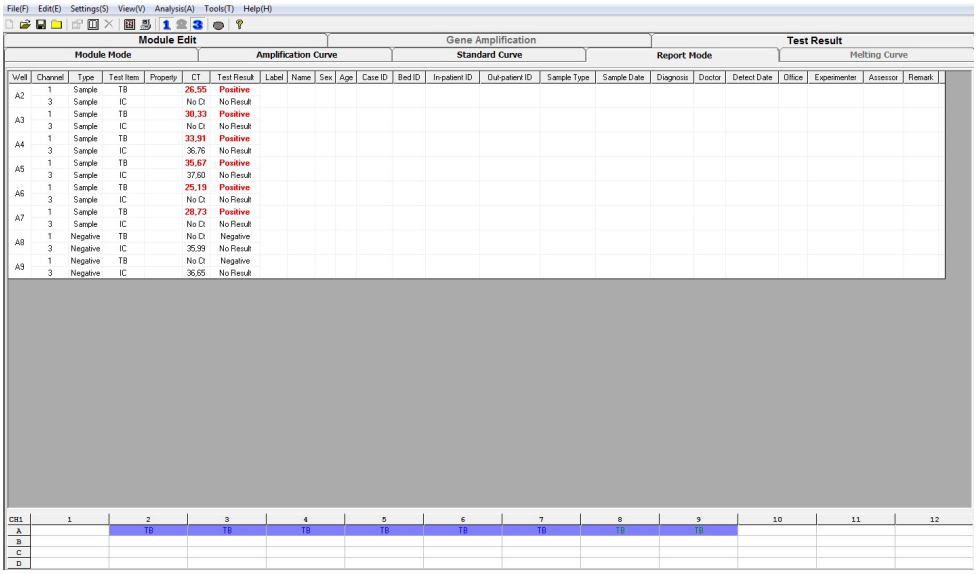

Fig. 6: A Report Mode Screen Showing the Results

The following table shows the possible results and their interpretation:

Signal detected in FAM filter pair	The sample contains MTBC DNA, the result is positive	No need to check the internal control since the sample is positive (high positive samples may suppress the signal from the internal control)
No signal in FAM, signal in Cy5	The MTBC DNA in the sample is not detectable	Signal from Cy5 filter pair rules out the possibility of PCR inhibition
No signal in FAM and Cy5	The diagnosis is inconclusive	No signal in Cy5 points out to PCR inhibition or to a problem in DNA isolation (See 11.Troubleshooting)

11. TROUBLESHOOTING

Please contact the manufacturer in case of a problem during a run.

Late or no signal from the FAM filter from the positive control

Wrong thermal protocol is chosen	Make sure that the correct thermal protocol is chosen.
Deterioration of the positive control	Don't use expired positive control or kit components. Follow the instructions for the storage of kit components (Section 3. Storage).

No signal from the internal control

Deterioration of the internal control or detection mix 2	Follow the instructions for the storage of kit components (See Section 3. Storage).
PCR inhibition	Make sure that you use the recommended DNA isolation method (See 9.3 DNA isolation).
DNA lost during isolation	Make sure that you use the recommended DNA isolation method (See 9.3 DNA isolation).

Signal from FAM Filter in the Negative Control

Contamination	Use filter-tips. Repeat PCR with new kit components.

The Threshold is Above Low Signals

The threshold should be manually adjusted	Using the mouse pull the threshold down until it cuts the low signals. Avoid the background and the signal from negative control.

Uneven Traces

Baseline cycles should be readjusted	Assign new baseline cycles manually and recalculate threshold cycles.

12. SPECIFICATIONS

12.1. Sensitivity

Analytical sensitivity may be expressed as the limit of detection: i.e. the smallest amount of the target marker that can be precisely detected. The detection limit of an individual analytical procedure is the lowest amount of nucleic acid in a sample which can be detected but not necessarily quantitated as an exact value. The analytical sensitivity or detection limit for NAT assays is expressed by the 95% positive cut-off value.

The analytical detection limit for Bosphore® MTBC v1 was found to be $1.28x10^1$ copies/ml (p=0.05). The sensitivity was determined using a dilution series of a quantitated DNA Control. The dilutions were tested in different runs in replicates. The results were analyzed by probit method.

12.2 Cross-Reactivity

To eliminate potential cross-reactivity, both assay design evidence and experimental studies were employed. Primer and probe sequences were checked for possible homology to other known pathogen sequences by sequence comparison analysis using database alignment. Samples of Chlamydia, Toxoplasma, CMV, EBV, Parvovirus B19, BKV with known high positivity were tested, and found negative.

12.3 Reproducibility

Reproducibility data (on C_T value basis) were obtained by the analysis of positive control of the Bosphore® MTBC Detection Kit v1. Test was performed in at least 4 replicates by 3 different operators, on multiple days, using 3 different lots. The resulting data is given in Table 1:

Table 1: Reproducibility Data.

MTBC (10^4 copies/ml)	Standard deviation	Variance	Coefficient of variation [%]
Intra-assay Variability N=4	0.043	0,0018	0,13
Inter-lot Variability N=3	0,070	0,0052	0,21
Inter-operator Variability N=3	0,017	0.0002	0.05
Total Inter-assay Variability N=5	0.043	0,0018	0,13

13. REFERENCES

1. K. E. Nelson, C. Williams, and N. Graham., Infectious Disease Epidemiology: Theory and Practice, July 15, 2000, p :653-655
2. Anonymous, Tuberculosis Fact Sheet, 2009 Update, World Health Organization.
3. Thierry Zozio, Caroline Allix, Selami Gunal, Genotyping of *Mycobacterium tuberculosis* clinical isolates in two cities of Turkey: Description of a new family of genotypes that is phylogeographically specific for Asia Minor, BMC Microbiol. 2005; 5: 44
4. Barry R. Bloom ,William N. Rom ,Stuart M. Garay , Tuberculosis: Pathogenesis, Protection, and Control ,Oct 1, 1994, p:47-49

14. SYMBOLS

Use by

Lot/Batch

Catalog number

Temperature limitation

Caution, consult accompanying documents

Manufacturer

In Vitro Diagnostic Medical Device

15. CONTACT INFORMATION

Anatolia Geneworks
Egitim Mh. Kasap Ismail Sk.
No:10/23 Kadikoy 34722
ISTANBUL-TURKEY
Phone: +90 216 330 04 55
Fax: +90 216 330 00 42
E-mail: info@anatoliageneworks.com
www.anatoliageneworks.com

Registered Trademarks: Anatolia Geneworks® Montania®, Magnesia® and Bosphore® are registered trademarks of Anatolia Tani ve Biyoteknoloji A.S.